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**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

97203974.7

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

**R C van Dijk**

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# Blatt 2 der Bescheinigung
# Sheet 2 of the certificate
# Page 2 de l'attestation

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Bezeichnung der Erfindung:
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Titre de l'invention:
Peptides, polypeptides and nucleic acids derived from eisenia foetida and their use in tumour therapy microbial infection, inflammation or immunology

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# PEPTIDES, POLYPEPTIDES AND NUCLEIC ACIDS DERIVED FROM *EISENIA FOETIDA*, AND THEIR USE IN TUMOUR THERAPY, MICROBIAL INFECTION, INFLAMMATION OR IMMUNOLOGY

The invention relates to *Eisenia foetida* polypeptides and peptides, particularly recombinant polypeptides, which are useful in tumour therapy, microbial infection, inflammation or immunology.

The invention also relates to a process for preparing the above-said polypeptides and peptides.

Furthermore, the invention concerns nucleic acids coding for said polypeptides and peptides.

Tumour Necrosis Factor α (TNF-α) is a multifunctional cytokine, produced in vertebrates, mainly by activated macrophages. *In vitro*, it has several biological effects, including killing of transformed cells and antiparasitic effects. Moreover, TNF-α has been shown to have a lectin-like property for the oligosaccharide ligands chitobiose and Man(α1,3)-Man(α1,6)-Man (1, 2). Recently, Lucas and co-workers (3) have mapped the lectin-like domain of TNF-α and have shown that the domain exerts trypanolytic activity on salivarian trypanosomes such as *Trypanosoma brucei.* The lectin-like activity of TNF-α is functionally involved in interactions with trypanosomes and possibly also with other pathogens.

The prophenoloxidase (proPO) activating system represents an important defence mechanism in a large variety of invertebrates (4, 5). This system is based on the recognition of bacterial antigens such as lipopolysaccharide (LPS), or peptidoglycan and β-1,3-glucan, present as major components of the cell wall of yeasts and fungi (6, 7). Generally, upon the recognition of such carbohydrat s proteinases cleave by limited proteolysis inactive proPO to its

active state, phenoloxidase (PO). The active enzyme catalyses the o-hydroxylation of monophenols as well as the oxidation of diphenols to quinones which are subsequently polymerised non-enzymatically to melanin. Melanin and its precursors involved in the proPO activating system have cytotoxic and antimicrobial properties and participate in a wide range of other biological activities including phagocytosis/opsonization, encapsulation/nodule formation, degranulation and wound healing (8-11).

The prophenoloxidase activating system has been detected both in protostomian and deuterostomian species. Though proPO activating system is well documented in arthropods, data in other protostomian groups are more scarce. In annelids, melanization reactions and formation of "brown bodies" or nodules have been described in polychaetes and oligochaetes (12-16). However, biochemical detection of PO activity was so far restricted to a few species with rather controversial results. While Smith and Söderhäll (17) failed to detect proPO system in the polychaete *Aphrodite aculeata* and *Arenicola marina*, Fischer (18), Valembois *et al.* (19), and Porchet-Hennerè and Vernet (15) have evidenced PO activity in *Lumbricus terrestris, Eisenia foetida andrei* and *Nereis diversicolor* respectively. More recently, using L-DOPA as substrate, a 30 kDa protein responsible for PO activity was identified in the coelomic fluid of *Lumbricus rubellus* (20). A report showing that the oxidative activity of the coelomic fluid of earthworms towards L-DOPA *in vitro* is not affected by trypsin but completely blocked by subtilisin reflects the importance of a correct proteolytic digestion as an initial step for inactive proPO activation (19).

Since the factor which recognises microbial carbohydrates and triggers the proPO system has not yet been d scribed in annelids (4, 5), investigations were initiated to identify such a molecule in the coelomic fluid (CF) of *E. Foetida*. Surprisingly, it is shown in this invention that a previously described 42 kDa cytolytic protein named CCF-1 (Coelomic Cytolytic Factor 1) (21) binds LPS and β-1,3 glucan and that the same protein is also responsible for the trypanolytic activity of the coelomic fluid. By combining the glucan and LPS binding capacity with the cytolytic and trypanolytic activity, the invertebrate factor resembles the vertebrate compound TNF-α and can therefore be considered as a primitive type of cytokine, which may be useful as an alternative for TNF-α. This idea is supported by the fact that an anti-TNF monoclonal antibody (anti-TNF/TIP) crossreacts with CCF-1, whereas an anti-CCF-1 monoclonal antibody (12C9) crossreacts with TNF-α. Moreover, in *E. foetida,* CCF-1 levels are increased after LPS treatment, which ressembles the TNF induction by LPS, noticed in vertebrates. Apart from the above described characteristics, it is shown that CCF-1 also participates in the proPO cascade of the coelomic fluid of *Eisenia foetida.*

Even more surprisingly, the cytolytic, trypanolytic and glucan-binding characteristics of the protein can be attributed to a small domain of 13 amino acids. Moreover, this isolated peptide of 13 amino acids is showing biological activity. The sequence of this peptide, however, is completely different from the TIP region of TNF-α, although it shares some functional characteristics.

It is an object of the current invention to provide new *Eisenia foetida* polypeptides and th ir corr sponding nucleic acids which can be used in the field of tumour therapy, microbial infection, inflammation or immunology.

It is another object of the invention to provide a nucleic acid coding for the peptide or polypeptide chains of biologically pure, active recombinant peptides which enable their preparation on large scale. When this nucleic acid, encoding for the (poly)peptide, is placed after an appropriate promoter, several host organisms, such as *E.coli, Bacillus sp., Streptomyces sp.,* yeast, fungi, insect cells, plant cells or mammalian cells can be used for the production of the recombinant protein. Alternatively, the peptides may be produced by chemical synthesis.

A peptide or polypeptide according to this invention is characterised by the fact that it contains at least 9 contiguous amino acids from sequence id. n°1.

One embodiment of the invention is given by the polypeptide of sequence id. n° 3.

According to another embodiment of the invention, the above defined peptides or polypeptides are exerting a trypanocidal or trypanolytical activity on *T. brucei* and/or *T. cruzi,* alone or preferably in combination with one of the following characteristics:

- exerting cytolytical activity
- exhibiting a β-1,3 glucan binding capacity and/or a LPS binding capacity.
- exerting opsonizing and/or hemolytic activity
- participating in the proPO cascad of *E. foetida.*

Another embodiment of the invention is the use of a peptide or polypeptide comprising at least 9 contiguous amino acids from sequence id. n° 1, such as e.g. the polypeptide given by sequence id. n° 3, for the manufacturing of a medicament to treat trypanosomal infection, bacterial infection or cancer. For the treatment of cancer, the peptide or polypeptide can be linked to a tumour specific antibody that directs the molecule to the tumour were the (poly)peptide can exert its cytolytical activity.

Another embodiment of the invention is the use of a peptide or polypeptide comprising at least 9 contiguous amino acids from sequence id. n°1, or a polypeptide comprising sequence id. n° 3 for the preparation of a medicament to treat trypanosomal infection, bacterial infection or cancer.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 A and 1B: Trypanolytic activity of peptides

(A) Trypanolysis in function of the time (0.0125 mg/ml peptide)

(B) Trypanolysis (after 3 hours) in function of the concentration.

S2B is a biotinylated, irrelevant peptide.

Figure 2: Trypanolytic activity of purified natural CCF-1 (nCCF-1) versus recombinant CCF-1 (rCCF-1), tested on *T. brucei*.

Figure 3A and 3B:

(A) Inhibition of rCCF-1-mediated trypanolytic activity by anti-CCF-1 and anti-TNF/TIP monoclonal antibodies, tested on *T. brucei.*

(B) Inhibition of rCCF-1-mediated trypanolytic activity by N,N'-diacetylchitobiose, as tested on *T. brucei.*

Figure 4: Trypanolytic activity of rCCF-1 and CF on *T. cruzi.*

Figure 5: Cytolytic activity of rCCF-1, as measured on L929 cells, in presence and absence of Actinomycin D (10 µg/ml)

Figure 6: Involvement of CCF-1 in the activation of PO. Level of L-DOPA oxidation, assessed after 6 hrs of incubation, are expressed as the ratio ± standard deviation of OD of the sample without and with proteinase inhibitor.

(A) entire coelomic fluid

(B) CCF-1-depleted coelomic fluid by pre-incubation on anti-CCF-1 immunoaffinity column

(C) CCF-1 depleted coelomic fluid supplemented with 0.5 µg/ml rCCF-1

(D) as (C), but with 1 µg/ml rCCF-1

(E) as (C), but with 2 µg/ml rCCF-1

(F) CCF-1 depleted coelomic fluid supplemented with 2 µg/ml bovine serum albumin

Laminarin or LPS were given at 2 µg/ml or 1 µg/ml respectively.

## EXAMPLES

### 1. purification and partial s qu ncing of CCF-1

CCF-1 was prepared and purified as described earlier (21). Analysis on 2 dimensional polyacryl amide gel electrophoresis (PAGE) confirmed the homogeneity of the CCF-1 preparation since a single spot was detected. A bulk preparation of immunoaffinity purified CCF-1 was separated on a preparative SDS-PAGE, blotted on PVDF problott and stained with amido black. 1/10$^{th}$ of the amount of the immobilised protein was N-terminal sequenced for 30 cycles. The N-terminal amino acid sequence of 30 residues is shown in Table 1. The remaining part of the CCF-1 containing PVDF band was used for internal sequence determination. To this end, tryptic digestion was performed according to the method of Fernandez *et al.* (22). After cleavage, the released peptides were separated on a reverse phase column (Vydac C4, 2.1 x 250 mm) and eluted with a linear gradient (0% to 70%) of acetonitrile in 0.1% trifluoroacetic acid. The column outlet was directly connected to a 1000 S diode assay detector (Applied Biosystems) and the most prominent peaks were used for amino acid sequencing. Purified peptides were sequenced using a pulsed-liquid model 477A sequenator (Applied Biosystems). The sequences of some of the peptides are shown in Table 1. From a number of peptide sequences degenerate PCR primers were deduced to identify CCF-1 cDNA from a cDNA library of *E. foetida.*

**2. trypanolytic activity of CCF-1**

*Coelomic fluid collection*

Coelomic fluid from adults *Eisenia foetida foetida* earthworms was obtained by puncturing the coelomic cavity with a glass micropipette in presence of protease inhibitor (Pefabloc, Boehringer, 10 mM). The pooled suspension was centrifuged (100 x g, 10 min), and the cell-free coelomic fluid after re-centrifugation (3000 x g, 10 min) was stored at -70°C until used.

*Monoclonal antibodies (mAb's)*

Generation of anti-CCF-1 monoclonal antibodies (12C9) was previously reported (21, 24). Isotype matched monoclonal antibody 15D3 (IgG1) against *Bandeiraea simplicifolia* B4 isolectin was prepared using standard procedures and used as control in trypanolysis assays.

*Parasites*

Pleiomorphic and monomorphic *Trypanosoma brucei* AnTat 1.1E clone, kindly were provided by Dr N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium).

*Trypanolytic assay*

Purified parasites were resuspended at 4 x $10^6$/ml PSG (PBS, consisting of 2.13 g NaCl , 8.45 g $Na_2HPO_4$ and 0.43 g $NaH_2PO_4$ per liter, supplemented with 1 % glucose). 100 μl of suspension were mixed with 100 μl of different concentrations of CF, CCF-1 or TNF–α (kindly provided by Innogenetics, Zwijnaarde, Belgium) in 96-well culture plate. Quantification of the trypanolysis was based on the determination of parasit viability using Ethidium homodimer (EthD-1, Molecular Probes). EthD-1 is excluded by

intact plasma membrane of live parasites. However, it enters the cells with damaged membrane and undergoes enhancement of fluor scence on binding nucleic acids, thereby producing a bright red fluorescence in dead cells. Briefly, after 4.5 H incubation at 30°C, 25 µl of 4.5 µM EthD-1 was added to the wells. The mean fluorescence intensity of triplicate cultures (±SD) was recorded 30 min later in a cytofluorimeter (Cytofluor II, PerSeptive Biosystems) using excitation / emission filters at 530 ± 25 / 645 ± 40 nm. A set of control was included in each experiments : (A) a parasite-free control to account for possible background fluorescence (B) a control of 100% lysis, prepared by treating the parasites with 10 µl 2 % saponin for about 10 min before adding EthD-1 (C) a control in which the trypanolytic component was replaced by PSG was considered as 0 % lysis. (B) and (C) were used to build a standard curve and the % lysis in test samples was estimated by linear regression. In some assays parasite lysis was assessed by light microscopy counting the remaining parasites. Background lysis, i.e. lysis of trypanosomes within 5 H incubation at 30°C in absence of lytic molecules, never exceeded 5-10%.

For inhibition experiments CCF-1 or TNF-α were pre-incubated 1 H at 30°C with 10 µg/ml of antibodies (anti-CCF1, anti-TIP or irrelevant mAb) or sugars (chitobiose, cellobiose, Sigma).

All experiments were repeated at least 3 times.

*Lysis of African trypanosomes*

CF of *E. foetida* exerts a trypanolytic activity on *T. brucei* parasites, that is not due to proteolysis since all experiments were performed in the presence of a

non-toxic serine proteinase inhibitor. Immunoaffinity purified CCF-1 exerts also a trypanolytic activity, 1000-times higher as compared to the total CF indicating an efficient enrichment. The trypanolytic activity of total CF as well as of CCF-1 is completly inhibited by the neutralising antibody mAb 12C9 (21) indicating that CCF-1 accounts for the total trypanolytic activity of CF (Table 2).

Since the trypanolytic activity of TNF-α is mediated by a lectin-like domain, it was tested whether CCF-1 exhibits similar features. N,N'-diacetylchitobiose, a potent inhibitor of the trypanolytic activity of TNF-α was found to inhibit also the trypanolytic activity of CCF-1. In contrast, cellobiose, that does not influence the trypanolytic activity of TNF-α, does not influence the trypanolytic activity of CCF-1 either. These results indicate that the CCF-1/trypanosome interaction involves a lectin-like activity. The lectin-like activity of TNF-α is mediated by a distinct domain of the molecule encompassing the amino acid sequence $T^{104}PEGAE^{109}$, designated as the TIP region of TNF-α (3). Since anti-TIP antibodies strongly inhibit the trypanolytic but not the cytolytic activity of TNF-α, the influence of such antibodies (polyclonal and monoclonal) was tested on the trypanolytic activity of CCF-1. According to the results, anti-TIP antibodies inhibit potently this activity (Table 3). Another monoclonal anti-TNF-α antibody (1F3F3) that neutralises potently the cytolytic (3) but weakly the trypanolytic activity of TNF-α does not interfere with CCF-1-mediated trypanolysis. These results suggest that CCF-1 and TNF-α share a similar region that mediates with interaction trypanosomes. This possibility was furth r on substantiated by following observations: (1) th neutralising anti-

CCF-1 mAb 12C9 inhibits th trypanocidal activity of TNF-α (Table 3), (2) monoclonal anti-TIP antibodies cross-react with CCF-1 in western blot and conversely the anti-CCF-1 mAb 12C9 binds on immobilised TNF-α, (3) both CCF-1 and TNF-α bind to immobilized chitobiose.

*Lysis of American trypanosomes*

It has been demonstrated that TNF-α exerts also trypanolytic activity on the American trypanosome *T. cruzi.* This trypanolytic activity can be inhibited by N,N'-diacetylchitobiose and by anti-TNF/TIP antibodies but not by the anti-TNF-α mAb 1F3F3 that neutralises the cytolytic effects of TNF-α.

In view of these results, we have tested whether CF is trypanolytic for *T. cruzi.* The results are summarised in Table 4. CF contains components that lyse *T. cruzi* and this lytic activity is substantially decreased by the neutralising anti-CCF-1 mAb 12C9 and by N,N'-diacetylchitobiose, but not by cellobiose. Hence the results indicate that CCF-1 is the major *T. cruzi* trypanolytic molecule of total CF and that this activity is again mediated via a lectin-like interaction similar to that one of TNF-α. Interestingly the *T. cruzi* trypanolytic activity of both TNF-α and CCF-1 could only be recorded on the trypomastigote but not the epimastigote forms of the parasite indicating that the susceptibility towards the lytic activity of TNF-α and CCF-1 is developmentally regulated.

**3. Cytolytic activity of CCF-1**

To test whether the cytolytic activity of CCF-1 is mediated by a similar or a different domain as that one utilised for the trypanolytic activity, CCF-1 was

preincubat d with N,N'-diacetylchitobiose or anti-TNF/TIP antibodies and test d in the L-929 lysis assay (21). The results are shown in Table 5. These results demonstrate clearly that the interaction of CCF-1 with L-929 cells is completely inhibited by treatment with N,N'-diacetylchitobiose and anti-TNF/TIP antibodies. This is in sharp contrast to the cytolytic activity of TNF-α that is not influenced by anti-TNF/TIP antibodies nor by N,N'-diacetylchitobiose. Hence, CCF-1 utilises its lectin-like domain to interact with trypanosomes and mammalian (L-929) cells and this interaction leads to cellular lysis.

**4. Identification of the trypanolytic domain of CCF-1**

The separated peptides obtained from HPLC purification of the tryptic digest of CCF-1 were analysed for reactivity with the anti-CCF-1 mAb via a dot spot assay. One peptide scored weakly positive with the 12C9 mAb and this peptide was sequenced. The obtained sequence is shown in sequence id. n°1. This peptide (termed CCF-1/TIP), which is clearly different from the TNF/TIP, was tested in trypanolysis assay and was found to be trypanolytic in a time- and dose-dependent way (Fig. 1). Furthermore, the CCF-1/TIP was reproducibly more trypanolytic than the TNF/TIP peptide, while irrelevant peptides were not or marginally trypanolytic (Fig. 1).

Profile analysis of the CCF-1/TIP sequence against the Swiss-prot database revealed that the CCF-1/TIF peptide contains a pattern that is unique for glycosyl hydrolases.

**5. Cloning and expression of CCF-1**

*Molecular cloning*

- RNA extraction

Earthworms were taken from compost and put on filter paper soaked with PBS for four days in order to clean their gut. After two days, Penicillin/Streptomycin (100U/100 µg/ml) were added to decrease the amount of micro-organisms in the worms and the worms were treated with LPS, a known inducer of CCF-1 synthesis. Three worms were frozen in liquid nitrogen, ground to powder and total RNA was prepared as described by Chomczynski and Sacchi (23). Poly $A^+$ RNA was isolated from the total RNA using the polyAtract system (Promega). Biotinylated-Oligo(dT) was added to the total RNA and allowed to bind the poly $A^+$ RNA. Using streptavidin-paramagnetic beads, the oligo(dT)-mRNA hybrids were captured and finally the mRNA was eluted. The mRNA isolated yielded 8 µg mRNA in 250 µl. After analysis on agarose gel, a smear of mRNA was observed and the rRNA was almost completely removed.

- cDNA library construction

cDNA was synthesised using a cDNA synthesis module (Amersham).

To make a cDNA library, the lambda zap II vector of Stratagene was used. Before inserting the cDNA in the lambda vector, *Eco*R I adaptors were ligated onto the ends of the cDNA. The adapted cDNA was size fractionated, phosphorylated and ligated in the lambda arms. The recombinant lambda DNA was packaged *in vitro* with Gigapack II Gold Packaging extracts

(Stratagene). *E. coli* (strain XL1-Blue MRF', Stratagene) was infected with the phage suspension for amplification and determination of the phage titer.

- Isolation of CCF-1 cDNA

Degenerate primers were deduced from a number of native earthworm CCF-1 peptide sequences in order to identify the CCF-1 cDNA from the *E. foetida* library. Combination of the following primers (5'TIACIGAITGGGAICAA/GTAT/CATIGTITGGCA3' and 5'AAIGTITGIAAITTG/ATCICCG/ATAG/ATTCCA3') resulted in a specific PCR fragment. A DIG labelled PCR product (DIG labelling mix, Boehringer, Mannheim) was subsequently used as a CCF-1 specific probe in plaque hybridisation. Positive plaques were isolated and the presence of CCF-1 cDNA was confirmed with PCR. In order to obtain pBluescript phagemids *in vivo* excision was performed according to Stratagene's recommendations.

- Sequence analysis of the CCF-1 cDNA

Since the 2500 bp *Eco*R I insert was too large to be sequenced in one run and since no information was available on the DNA sequence of the insert, deletions were made of the clone. With a Erase-a-base system (Promega) a series of deletions of the *Eco*R I insert of the pBluescript vector were made in which the insert was each time 250 bp smaller. Using these deletions it was possible to sequence the 2500 bp *Eco*R I insert of the pBluescript vector.

- Analysis of CCF-1 cDNA

The sequencing revealed that the *Eco*R I insert was 2682 bp of length. In the insert two open reading frames (ORF) were recognised. One ORF showed a strong homology with cDNA of the Myosin ssential light chain of the

earthworm *Lumbricus terrestris*. In the second ORF, all identified sequences, including the CCF-1/TIP sequence, of the natural CCF-1 of *E. foetida* wer present. These results indicate that the second ORF is the compl te cDNA of CCF-1. Further analysis of the CCF-1 cDNA showed that the full length cDNA was isolated with a length of 1115 bp, starting with an ATG initiation codon and ending with a TGA stop codon (sequence id. n° 2). The deduced amino acid sequence contains an eukaryotic signal sequence of 17 amino acids, indicating that the mature CCF-1 starts at amino acid 18. The deduced amino acid sequence contains 3 cysteins, possibly forming one sulphur bridge. There are no N-glycosylation sites present, excluding N-glycosylation of the protein. Since the molecular weight of the natural CCF-1 and of the deduced amino acid sequence are similar, there are probably no O-glycosylations present.

*Expression of recombinant CCF-1*

- construction of the pIGRI-CCF-1 and pIGRHISA-CCF-1 vector

The c-DNA sequence encoding for mature CCF-1 (mCCF1) was amplified by PCR using PWO polymerase (Boehringer Mannheim) and the pBluescript phagemid as template. The primers were designed so that after PCR, the m-CCF-1 cDNA contained *Bam*H I/. *Nsi* I sites at the 5' end (GGGGATCCATGCATTCACCGACTGGGATCAATATCAC) and a *Sal* I site at the 3' end (CCGTCGACTCAGTTGCGCTTGTAGACTCG). Hence, after cutting the PCR product with *Nsi* I and blunting the sticky ends, the first codon of the mCCF-1 was blunt-end available for ligation.

The *Bam*H I-*Sal* I fragment was subcloned in pBluescript (pBSmCCF-1) and checked by sequencing. A *Nsi* I blunt d -*Sal* I fragment containing the mCCF-1 cDNA from pBSmCCF-1 was cloned into pIGRI2 (resulting in pIGRI-CCF-1) and pIGRHISA (resulting in pIGRHISA-CCF-1). pIGRI2 is a vector for intracellular expression of mature proteins and pIGRHISA is a vector for expression of proteins including a His-tag. In the latter vector, the mCCF-1 cDNA sequence is preceded by an amino-terminal His-tag and an enterokinase cleavage site. After transformation in the *E. coli* strain MC1061 pAcI the clones were ready for induction.

- induction of recombinant CCF-1 protein (rCCF-1)

Since CCF-1 in both constructs is under control of the $P_L$ promoter, cultures were grown at 32°C and induced at 42°C at an $OD_{600}$ of 0.7. Total cell lysates showed an extra band after induction of the cultures for both the mature and the His-tag recombinant protein. The band after induction of pIGRI-CCF-1 had a size of approximately 42 kDa and the band after induction of pIGRHIA-CCF-1 had a size of approximately 44 kDa. High expression of recombinant protein was obtained and both bands were the most prominent ones of the total cell lysates.

In order to prove that the induced *E. coli* proteins of 42 or 44 kDa were CCF-1, a western blot was performed using mAb 12C9 and mAb against TNF/TIP. Both recombinant proteins showed a clear band on western blot incubated with 12C9 mAb as well as with anti-TNF/TIP mAb. Moreover, the binding capacity towards laminarin, LPS and N,N'-diacetylchitobiose of the proteins,

produced by *E. coli*, is comparable with the capacity of natural CCF-1. These data show clearly that the induced protein (with and without His-tag) is CCF-1.

- purification of rCCF-1

One litre of pIGRHISA-CCF-1 transformed bacterial culture was resuspended in PBS and sonicated. After sonication, the pellet of *E.coli* transformed with rCCF-1 cDNA cloned into pIGRHISA plasmid was solubilised in Urea (8 M in 20 mM Tris pH 8.5 - 50 mM NaCl), applied on 2.5 ml Ni-NTA agarose resin (Qiagen) and renatured by a linear decreasing gradient of Urea while rCCF-1 was bound on the column. Elutions were performed by imidazole (300 mM in 20 mM Tris pH 8.5 - 50 mM NaCl) and imidazole was removed by extensive dialysis against PBS pH 8.0. rCCF-1 was further purify to homogeneity by immunoaffinity on anti-CCF-1 12C9 or anti-TIP 24C11 monoclonal antibody columns.

**6. Biological activity of rCCF-1**

*Trypanolytic activity*

The trypanolytic activity was determined as described under point 2.

rCCF-1 is trypanolytic for the African trypanosome *T. brucei* in a dose dependent manner (Fig. 2). The trypanolytic activity of rCCF-1 can be inhibited by anti-CCF-1 and anti-TNF/TIP mAb's (Fig. 3A). Furthermore N,N'-diacteylchitobiose inhibits potently trypanolytic activity of rCCF-1 (Fig. 3B). These data corroborate the findings that CCF-1 shares a trypanolytic, lectin-like domain with TNF-$\alpha$.

rCCF-1 exerts also a trypanolytic activity on the American trypanosome *T. cruzi* (Fig. 4) .

*Cytolytic activity*

Cytolytic activity of rCCF-1 was tested as described (21). rCCF-1 is clearly cytolytic, especially in the presence of actinomycin D, a well known enhancer of the cytolytic activity of TNF-$\alpha$ (Fig. 5).

*Involvement in prophenoloxidase activation*

The level of prophenoloxidase system activation was assessed according to Valembois *et al.* (19). Briefly, 50 µl of the coelomic fluid (without or with 1 mM Pefabloc {serine proteinase inhibitor}, Boehringer), 25 µl 0.1 M Tris pH 8 containing 50 mM $Ca^{2+}$ and 10 µl L-DOPA (3-(3,4-dihidroxylphenyl)-L-alanine {Fluka}, final concentration 1.5 mM) were incubated at room temperature for different time intervals in the absence or presence of soluble LPS from *E. coli* (Difco, 1 µg/ml) or laminarin (Sigma, 2 µg/ml). The oxidation of L-DOPA was measured at 492 nm and expressed as the ratio between the values without and with Pefabloc. To confirm the role of glucan- or LPS-binding protein in proPO activation the coelomic fluid was incubated with anti-CCF-1 (12C9) immunoaffinity column (Affi-Gel, Bio-Rad) for 1 H at 4 °C. The samples were centrifuged and the depleted coelemic fluid was used in L-DOPA oxidation test as described above. To reconstitute the proPO activating cascade, rCCF-1 (0.5, 1 and 2 µg/ml) was added to CCF-1 depleted co lomic fluid before testing L-DOPA oxidation.

In order to confirm that CCF-1 glucan- and LPS-binding protein is involved in the activation of proPO cascade, CCF-1 was removed from the entire coelomic fluid by preincubation with insoluble glucan or anti-CCF-1 antibody column. This results in a significant decrease of the oxidative activity of the coelomic fluid even in presence of laminarin or LPS (Fig. 6). The activity of the CCF-1 depleted coelomic fluid can be completely recovered by addition of rCCF-1.

**7.In vivo activity of CCF-1**

*CCF-1 purification*

Native CCF-1 (CCF-1) and recombinant CCF-1 (rCCF-1) were purified as described above. Both CCF-1 and rCCF-1 were finally suspended in PBS (pH8)

*Parasites*

Pleiomorphic and monomorphic *Trypanosoma brucei* AnTat 1.1E clone, kindly were provided by Dr N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium). Female (C57Bl/6 x Balb/c) F1 mice (2 months old, Bantin and Kingman, North Humberside, UK) were inoculated intraperitoneally with 2 x $10^3$ viable bloodstream form parasites. Animals were bled on heparin (20 U/ml) at the first peak of parasitaemia. Blood was diluted in an equal volume PSG and purified over a DE52 cellulose ion exchange chromatography column (Whatman) using PSG for equilibration and elution (25). Purified trypanosomes were subsequently washed in PSG (1000 x g, 20 min).

*Antibodies*

Generation of anti-CCF-1 monoclonal antibodies (12C9) was previously reported (21, 24). Isotype matched monoclonal antibody 15D3 (IgG1) against *Bandeiraea simplicifolia* B4 isolectin was used as control in trypanolysis assays and during in vivo antibody treatment of *T.brucei* infected mice.

*Trypanolytic assay*

The trypanolytic assay was carried out as described above.

*Antibody or rCCF-1 treatment during Trypanosome infection*

Groups of (C57Bl/6 x Balb/c) F1 mice received one intraperitoneal injection of 50 μg purified antibody (anti-CCF-1, anti-TIP or control mAb) or 200 μg rCCF-1 24 H before infection with 2 x $10^3$ pleomorphic AnTat 1.1 parasites. Parasitaemia was monitored by tail blood puncture every two to four days using a counting chamber. Before treatment of mice, antibodies were adsorbed on polymyxin beads (Sigma) to avoid LPS contamination. The presence of LPS in rCCF-1 preparation was excluded by E-Toxate test (Sigma).

*Anti-CCF-1 treatment increases parasite load in T.brucei-infected mice.*

Treatment of *T.brucei*-infected mice with TNF/TIP-specific antibody resulted in a dramatic increase in the number of parasites during the first peak of parasitaemia (24). In view of the similarity between the lectin-like domain of CCF-1 and TNF–α, we evaluated whether anti-CCF-1 monoclonal antibody treatment before *T.brucei* infection influenced the parasitaemia (Table 6). Compared to untreated (not shown) or control antibody-treat d mice, *T.brucei*-infected animals treated with CCF-1-specific antibodi s show a

substantial increase in the number of parasit s in the blood ($p<0.05$).

However the increase in parasitaemia following anti-CCF-1 treatment is lower than in anti-TNF/TIP-treated mice (not shown).

*CCF-1 treatment impairs T.brucei proliferation within infected mice.*

The trypanolytic activity of CCF-1 in vitro raised the possibility that CCF-1 treatment would influence the development of *T.brucei* in infected mice. As shown in Table 7, animals treated with rCCF-1 before trypanosome infection control more efficiently the replication of the parasite in the blood. The first peak of parasitaemia is reduced approximately by 30% in such mice as compared to untreated animals ($p<0.05$).

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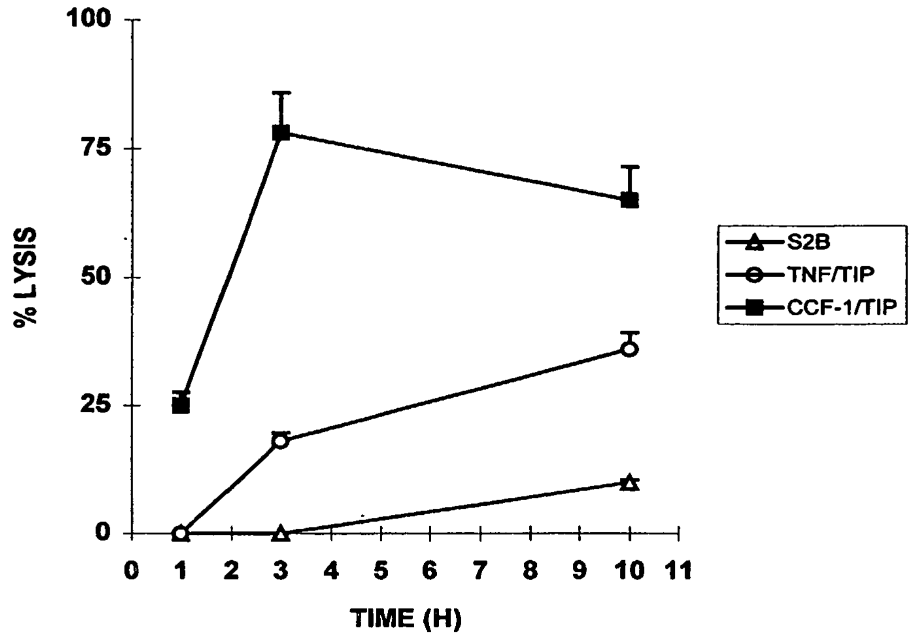
**CLAIMS**

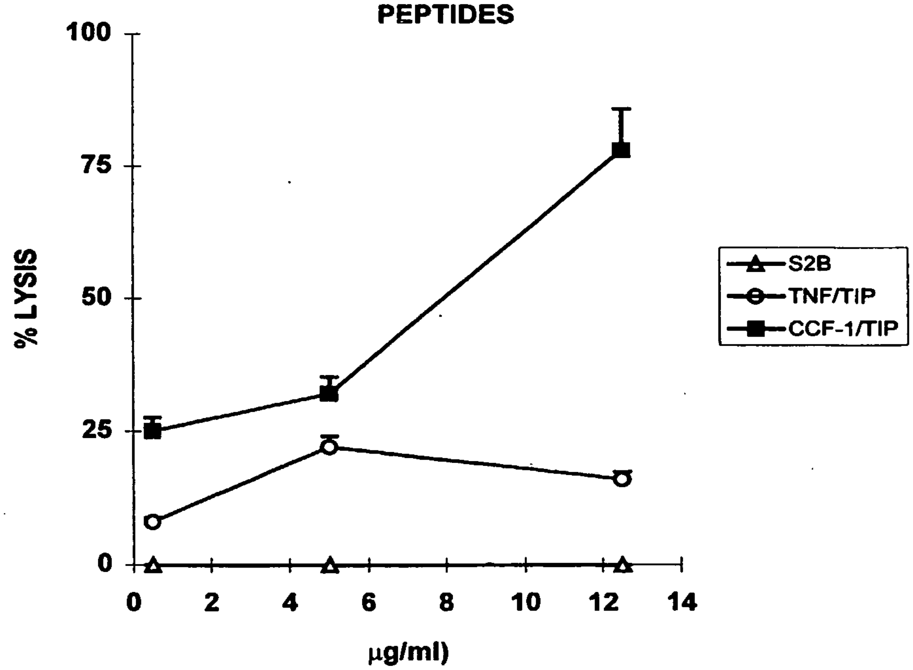
1. A peptide or polypeptide, comprising at least 9 contiguous amino acids from sequence id n°1
2. A polypeptide comprising the amino acid sequence id. n°3.
3. A peptide or polypeptide, according to claim 1 or 2, exhibiting trypanolytic activity, preferably in combination with cytolytic and/or glucan binding and/or LPS binding and/or opsonizing activity.
4. An isolated DNA sequence encoding a peptide or polypeptide, according to any of the claims 1-3.
5. An isolated DNA sequence according to claim 4, comprising the sequence id. n° 2, or parts of it.
6. A recombinant expression vector comprising a DNA sequence according to claim 4 or 5.
7. A host cell transformed or transfected with an expression vector according to claim 6.
8. The host cell of claim 7, wherein the host cell is selected from the group consisting of *E. Coli, Bacillus sp., Streptomyces sp.,* yeast, fungi, insect cells, plant cells or mammalian cells.
9. The host cell of claim 8, wherein the host cell is *E. Coli.*
10. A pharmaceutical preparation comprising a peptide or polypeptide according to claim 1, 2 or 3.
11. Use of a peptide or polypeptide, according to claim 1, 2 or 3, for the preparation of a medicament to treat trypanosomal infection, bacterial infection or cancer.

Figure 1

A : KINETIC OF LYSIS INDUCED BY PEPTIDES




B : DOSE-DEPENDENT LYSIS MEDIATED BY PEPTIDES



DEAD

17.12.97

2|6

97203974



Figure 2

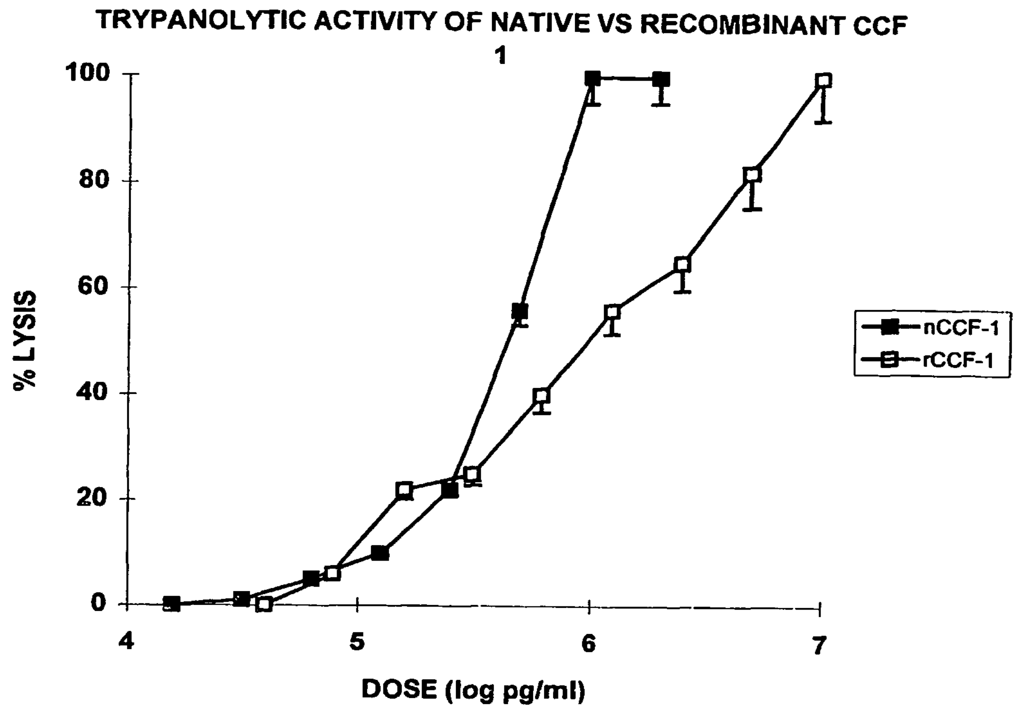


Figure 3 A

**INHIBITION OF rCCF-1- MEDIATED TRYPANOLYSIS BY ANTI-CCF-1 OR ANTI-TNF TIP ANTIBODIES**

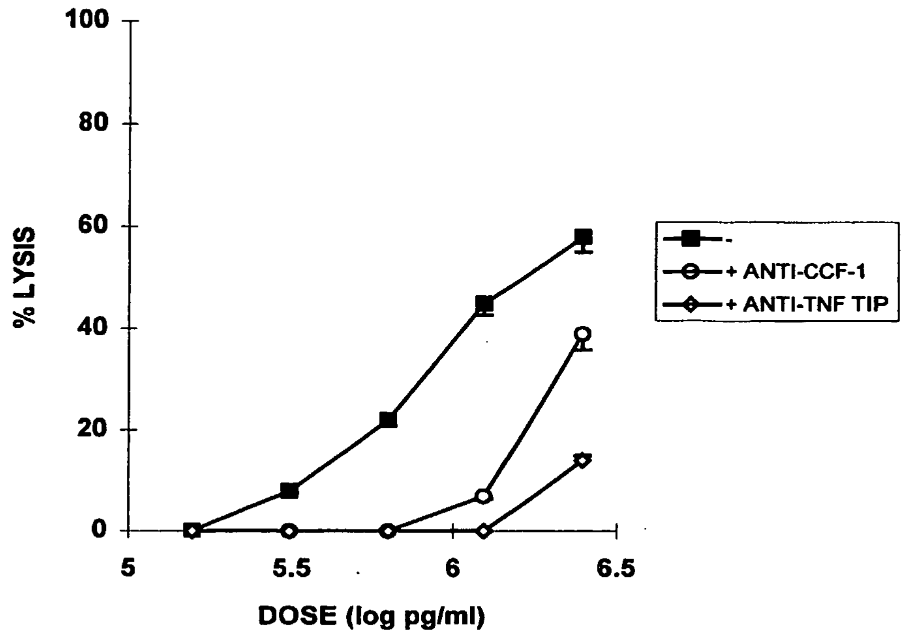
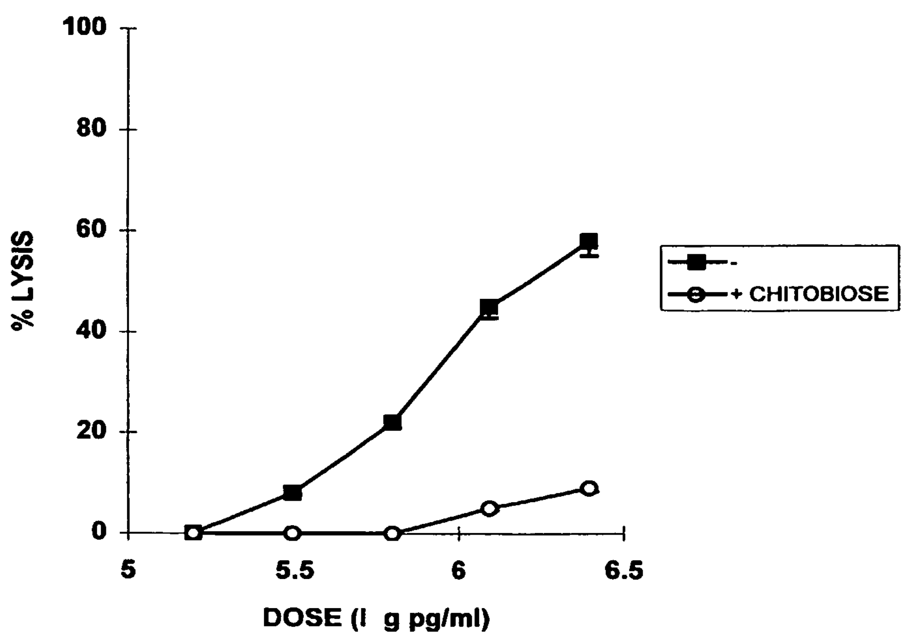



Figure 3 B

**INHIBITION OF rCCF-1- MEDIATED TRYPANOLYSIS BY CHITOBIOSE**



30. 17·12·97

46





Figure 4

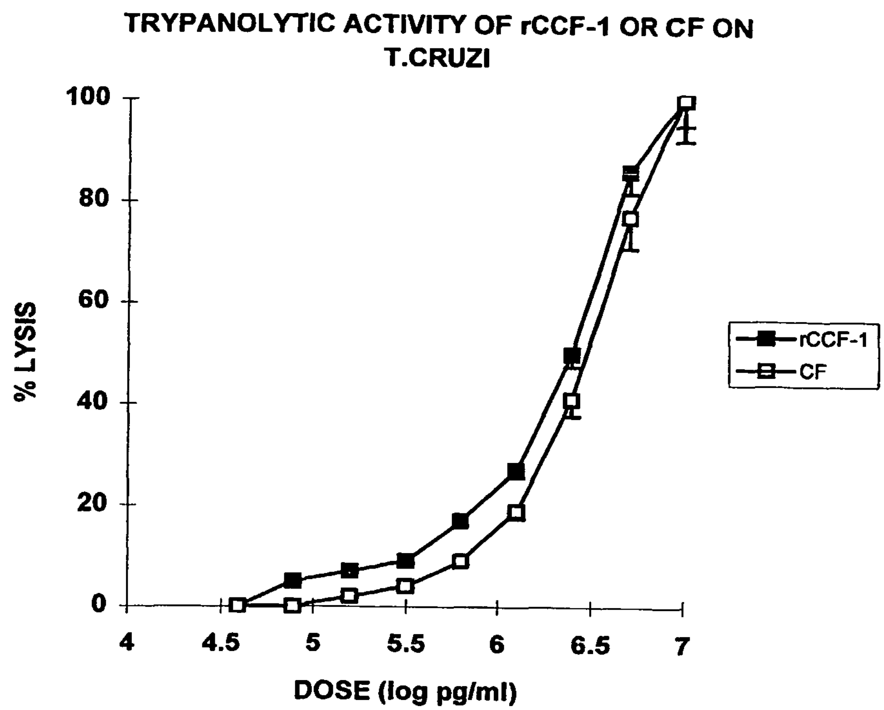


Figure 5

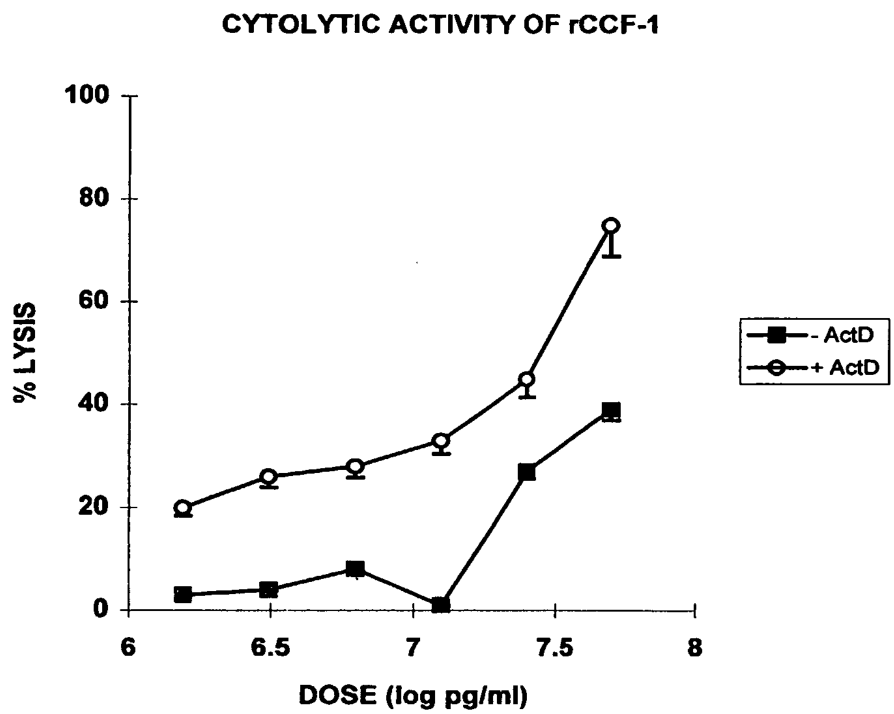

CYTOLYTIC ACTIVITY OF rCCF-1
100
80
60
40
20
0
% LYSIS
6
6.5
7
7.5
8
DOSE (log pg/ml)
- ActD
+ ActD

Figure 6

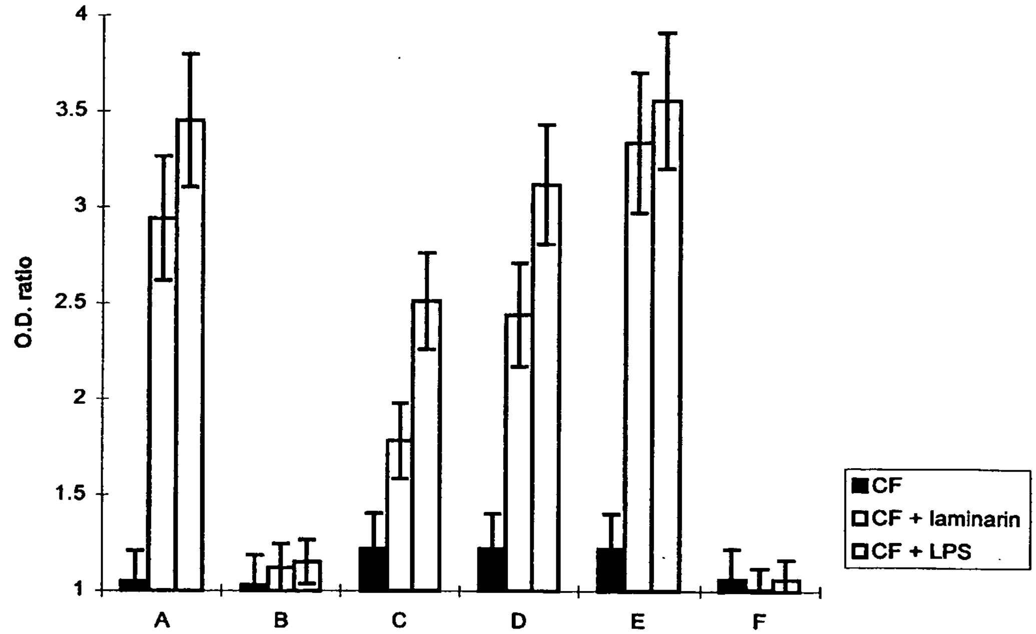


**Table 1 : aminoacid sequ nc of CCF-1 and TNF/TIP peptides**

| Peptide | Aminoacid sequence[a] |
|---|---|
| CCF-1.1 | N-terminus : NH2-FTDWDQYHIVWQDEFDYFDGAKWQHEVTAT-COOH |
| CCF-1.2 | (R,K) ↓ NH2-VYK-COOH |
| CCF-1.4 | (R,K) ↓ NH2-NTGGEFLGIQK-COOH |
| CCF-1.5 | (R,K) ↓ NH2-MGSTMHWGPGWDDNER-COOH |
| CCF-1.8 | (R,K) ↓ NH2-YWLTSLPK-COOH |
| CCF-1.10 (CCF-1/TIP) | (R,K) ↓ NH2-SGEIDIIETIGNR-COOH |
| TNF/TIP | TPEGAEA |

**Table 2 : trypanolytic activity of CF and CCF-1.**

| CF tested[a] | Neutralizing antibody[d] (12C9) | % Trypanolysis | % Inhibition |
|---|---|---|---|
| 1. Total CF[b] | - | 97 | |
| | + | 10 | 90 |
| 2. CF flow through[b] (irrelevant IgG column) | - | 94 | |
| | + | 7 | 93 |
| 3. CF flow through[b] (12C9 column) | - | 30 | |
| | + | 2 | 94 |
| 4. Eluate (CCF-1)[c] (12C9 column) | - | 42 | |
| | + | 0 | 100 |

a : CF and CF subfractions were purified by immunoaffinity on irrelevant IgG or 12C9 column and tested for trypanolytic activity in the trypanolysis assay (% trypanolysis was recorded after 2 H).

b : Concentration used = 1 mg/ml.

c : Concentration used = 4 µg/ml.

d : 12C9 antibody was added at a concentration of 10 µg/ml.

**Tabl 3 : inhibition of th trypanolytic activity (*T. brucei*) of CCF-1 and TNF-α by antibodies and carbohydrat s**

| Inhibitor[a] | CCF-1 mediated trypanolysis[b] | | TNF-α mediated trypanolysis[c] | |
|---|---|---|---|---|
| | % Lysis | % Inhibition | % Lysis | % Inhibition |
| None | 42 | - | 41 | - |
| N,N-diacetylchitobiose | 3 | 73 | 0 | 100 |
| Cellobiose | 49 | 0 | 41 | 0 |
| Polyclonal anti-TNF/TIP | 0 | 100 | 0 | 100 |
| Polyclonal IgG control | 46 | 0 | 43 | 0 |
| Monoclonal anti-TNF/TIP | 0 | 100 | 0 | 100 |
| Monoclonal IgG control | 49 | 0 | 41 | 0 |
| Monoclonal anti-CCF-1(12C9) | 0 | 100 | 1 | 98 |
| Monoclonal anti-TNF(1F31F3) | 44 | 0 | 41 | 0 |

a : Inhibitors were added at a final concentration of 10 μg/ml.

b : CCF-1 was added in the trypanolysis assay at a final concentration of 4 μg/ml.

c : TNF-α was added in the trypanolysis assay at a final concentration of 1.000 U/ml.

**Tabl 4 : inhibition of the trypanolytic activity (*T. cruzi*) of CF by antibodies and carbohydrat s**

| Inhibitor[a] | CF-1 mediated trypanolysis[b] | |
|---|---|---|
| | % Lysis | % Inhibition |
| None | 62 | - |
| N,N'-diacetylchitobiose | 19 | 70 |
| Cellobiose | 67 | 0 |
| Monoclonal anti-CCF-1(12C9) | 30 | 52 |
| Monoclonal IgG control | 67 | 0 |

a : Inhibitors were added at a final concentration of 10 μg/ml.

b : CF was added in the trypanolysis assay at a final dilution of 1 : 4.000.

**Tabl 5 : inhibiti n of the cytolytic activity of CCF-1 (L929) by antibodi s and carbohydrates**

| Inhibitor[a] | CCF-1 mediated cytolysis[b] | |
|---|---|---|
| | % Lysis | % Inhibition |
| Experiment 1 | | |
| None | 72 | - |
| N,N'-diacetylchitobiose | 0 | 100 |
| Monoclonal anti-CCF-1(12C9) | 0 | 100 |
| Monoclonal anti-TNF/TIP | 0 | 100 |
| Experiment 2 | | |
| None | 66 | - |
| Monoclonal anti-CCF-1(12C9) | 14 | 79 |
| Monoclonal anti-CCF-1(7F1) | 0 | 100 |
| Monoclonal anti-CCF-1(6H1) | 0 | 100 |

a : Inhibitors were added at a final concentration of 10 µg/ml

b : CCF-1 was added in the L929 cytolysis assay at a final concentration of 4 µg/ml

**Tabl 6 : parasita mia in mic treat d with anti-CCF-1 mAbs (group of 10 mic )**

| | Parasites x $10^6$/ml | |
|---|---|---|
| Day pi | Control mAb-treated | anti-CCF-1 treated |
| 3 | 104 | 135 |
| 4 | 129 | 194 |
| 5 | 64 | 84 |
| 6 | 2 | 2 |

**Tabl 7 : parasitaemia in untreated or CCF-1-treat d mic (group of 4 mice)**

| | Parasites x $10^6$/ml | |
|---|---|---|
| Day pi | untreated | rCCF-1 treated |
| 3 | 207 | 142 |
| 4 | 211 | 143 |
| 5 | 102 | 104 |
| 6 | 6 | 1.2 |

**Abstract**

The invention concerns *Eisenia foetida* polypeptides and peptides, particularly recombinant polypeptides, which are useful in tumour therapy, microbial infection, inflammation or immunology.

The invention also relates to a process for preparing the above-mentioned polypeptides and peptides.

Furthermore,the invention concerns nucleic acids coding for said polypeptides and peptides.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Vlaams Interuniversitair Instituut voor Biotechnologie
(B) STREET: Rijvisschestraat 118 box 1
(C) CITY: Zwijnaarde
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-9052
(G) TELEPHONE: +3292446611
(H) TELEFAX: +3292446610

(ii) TITLE OF INVENTION: Peptides, polypeptides and nucleic acids, derived from Eisenia foetida, and their use in tumour therapy, microbial infection, inflammation or immunology

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eisenia foetida

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Ser Gly Glu Ile Asp Ile Ile Glu Thr Ile Gly Asn Arg
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1155 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eisenia foetida

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION:1..51

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:52..1152

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1152

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GCC TTC ACC GAC TGG GAT CAA TAT CAC ATC GTC TGG CAG GAC GAA TTC 96
Ala Phe Thr Asp Trp Asp Gln Tyr His Ile Val Trp Gln Asp Glu Phe
1 5 10 15

GAT TAC TTT GAT GGC GCG AAG TGG CAA CAT GAG GTC ACA GCA ACT GGC 144
Asp Tyr Phe Asp Gly Ala Lys Trp Gln His Glu Val Thr Ala Thr Gly
20 25 30

GGA GGG AAC AGC GAA TTC CAA CTG TAC ACA CAG GAT GGG GCC AAC AGC 192
Gly Gly Asn Ser Glu Phe Gln Leu Tyr Thr Gln Asp Gly Ala Asn Ser
35 40 45

TTC GTT CGA GAT GGA AAG CTT TTC ATT AAG CCG ACG TTG CTG GCT GAC 240
Phe Val Arg Asp Gly Lys Leu Phe Ile Lys Pro Thr Leu Leu Ala Asp
50 55 60

AAC ATC AAC CCA CAG ACG GGT GCG CCA TTT GGA ACC GAT TTC ATG TAC 288
Asn Ile Asn Pro Gln Thr Gly Ala Pro Phe Gly Thr Asp Phe Met Tyr
65 70 75

AAT GGA GTT CTA GAT GTC TGG GCT ATG TAC GGG GCC TGC ACG AAT ACG 336
Asn Gly Val Leu Asp Val Trp Ala Met Tyr Gly Ala Cys Thr Asn Thr
80 85 90 95

GAC AAC AAC GGA TGC TAC AGG ACG GGA GCC GCT GGC GAC ATT CCA CCG 384
Asp Asn Asn Gly Cys Tyr Arg Thr Gly Ala Ala Gly Asp Ile Pro Pro

100 105 110

GCC ATG TCG GCA CGA GTT CGA ACC TTC CAG AAA TAC AGC TTC ACC CAC 432
Ala Met Ser Ala Arg Val Arg Thr Phe Gln Lys Tyr Ser Phe Thr His
115 120 125

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Gly Arg Val Val Val His Ala Lys Met Pro Val Gly Asp Trp Leu Trp
130 135 140

CCA GCC ATT TGG ATG TTG CCG GAG GAT TGG GTC TAT GGC GGA TGG CCT 528
Pro Ala Ile Trp Met Leu Pro Glu Asp Trp Val Tyr Gly Gly Trp Pro
145 150 155

CGA TCG GGC GAG ATC GAC ATC ATT GAA ACA ATC GGC AAC CGA GAT TTC 576
Arg Ser Gly Glu Ile Asp Ile Ile Glu Thr Ile Gly Asn Arg Asp Phe
160 165 170 175

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Lys Asn Thr Gly Gly Glu Phe Leu Gly Ile Gln Lys Met Gly Ser Thr
180 185 190

ATG CAC TGG GGT CCA GGA TGG GAC GAC AAC CGA TAC TGG CTG ACC AGC 672
Met His Trp Gly Pro Gly Trp Asp Asp Asn Arg Tyr Trp Leu Thr Ser
195 200 205

CTT CCG AAA CAC TCA GAC GAT TGG AAC TAC GGT GAC AAC TTC CAC ACG 720
Leu Pro Lys His Ser Asp Asp Trp Asn Tyr Gly Asp Asn Phe His Thr
210 215 220

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225 230 235

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Glu Asn Gln Ala Leu Leu Asp Val Pro Tyr Pro Leu Ile Asp Ala Asn
240 245 250 255

CCA TGG TGG GTG GAT TTC TGG GAG TGG GGA AAG CCC TGG CTT CCT CAA 864
Pro Trp Trp Val Asp Phe Trp Glu Trp Gly Lys Pro Trp Leu Pro Gln
260 265 270

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290 295 300

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305 310 315

CAG AAG CCG TGG AGC AAT GGG GAC TGG TAC AAC GAT GCA ATG AGG AAA 1056
Gln Lys Pro Trp Ser Asn Gly Asp Trp Tyr Asn Asp Ala Met Arg Lys
320 325 330 335

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Phe Phe Asp Ala Arg Gly Asn Trp Lys Trp Thr Trp Asp Asp Glu Gly
340 345 350

GAC AAC AAT GCC ATG CAG GTC GAT TAC ATC CGA GTC TAC AAG CGC AAC 1152
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355 360 365

TGA 1155

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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1 5 10 15

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Gly Gly Asn Ser Glu Phe Gln Leu Tyr Thr Gln Asp Gly Ala Asn Ser
35 40 45

Phe Val Arg Asp Gly Lys Leu Phe Ile Lys Pro Thr Leu Leu Ala Asp
50 55 60

Asn Ile Asn Pro Gln Thr Gly Ala Pro Phe Gly Thr Asp Phe Met Tyr
65 70 75

Asn Gly Val Leu Asp Val Trp Ala Met Tyr Gly Ala Cys Thr Asn Thr
80 85 90 95

Asp Asn Asn Gly Cys Tyr Arg Thr Gly Ala Ala Gly Asp Ile Pro Pro
100 105 110

Ala Met Ser Ala Arg Val Arg Thr Phe Gln Lys Tyr Ser Phe Thr His
115 120 125

Gly Arg Val Val Val His Ala Lys Met Pro Val Gly Asp Trp Leu Trp
130 135 140

Pro Ala Ile Trp Met Leu Pro Glu Asp Trp Val Tyr Gly Gly Trp Pro
145 150 155

Arg Ser Gly Glu Ile Asp Ile Ile Glu Thr Ile Gly Asn Arg Asp Phe
160 165 170 175

Lys Asn Thr Gly Gly Glu Phe Leu Gly Ile Gln Lys Met Gly Ser Thr

180 185 190

Met His Trp Gly Pro Gly Trp Asp Asp Asn Arg Tyr Trp Leu Thr Ser
195 200 205

Leu Pro Lys His Ser Asp Asp Trp Asn Tyr Gly Asp Asn Phe His Thr
210 215 220

Phe Trp Phe Asp Trp Ser Pro Asn Gly Leu Arg Phe Phe Val Asp Asp
225 230 235

Glu Asn Gln Ala Leu Leu Asp Val Pro Tyr Pro Leu Ile Asp Ala Asn
240 245 250 255

Pro Trp Trp Val Asp Phe Trp Glu Trp Gly Lys Pro Trp Leu Pro Gln
260 265 270

Tyr Glu Asn Asp Asn Pro Trp Ala Gly Gly Thr Asn Leu Ala Pro Phe
275 280 285

Asp Gln Asn Phe His Phe Ile Leu Asn Val Ala Val Gly Gly Thr Asn
290 295 300

Gly Phe Ile Pro Asp Gly Cys Ile Asn Arg Gly Gly Asp Pro Ala Leu
305 310 315

Gln Lys Pro Trp Ser Asn Gly Asp Trp Tyr Asn Asp Ala Met Arg Lys
320 325 330 335

Phe Phe Asp Ala Arg Gly Asn Trp Lys Trp Thr Trp Asp Asp Glu Gly
340 345 350

Asp Asn Asn Ala Met Gln Val Asp Tyr Ile Arg Val Tyr Lys Arg Asn
355 360 365